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The *BRCA2* tumor suppressor gene has been suggested to play an important role in DNA repair and maintaining genome integrity. This suggestion, however, is mainly based on results obtained from studying mouse embryonic cells. The importance of BRCA2 in maintaining genome integrity of human somatic cells is not very clear. We have completed the Task 1, generation of Capan-1 derivatives that conditionally express wild type BRCA2. We have also carried out the first part of the Task 3, characterization of Capan-1 derivatives to genotoxic agents. Our results showed that there was no detectable difference in the sensitivity to _ radiation and DNA damaging chemicals between Capan-1 cells that express the wild-type BRCA2 and those do not. We have performed Task 2, generation of MCF7 and MCF-12A derivatives that do not express BRCA2. We have attempted the antisense cDNA approach but did not obtain any clone that expressed reduced level of BRCA2. In order to disrupt the *BRCA2* gene, we have constructed a somatic gene-targeting plasmid. We have also investigated using siRNA to reduce BRCA2 level. We have succeeded in reducing BRCA2 level by transfecting MCF7 cells with synthetic siRNA. We are establishing plasmid-based siRNA expression system and we will use it to generate cells expressing reduced level of BRCA2.

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## Introduction

People carrying germline mutations of the breast cancer susceptibility gene *BRCA2* have increased risk for breast, ovarian, pancreatic and other types of cancer (1-3). Mouse cells lacking a functional *Brca2* gene are deficient in repairing DNA damage (4-8). Capan-1, a human pancreatic cancer cell line, is the only human cell line known to not express wild-type BRCA2. Capan-1 cells carry only a mutant *BRCA2* (6174delT) and expresses a truncated BRCA2 protein (9-12). The *BRCA2* 6174delT mutation is one that found frequently in Ashkenazi Jews and one that clearly predisposes its carriers to a variety of cancers (13-18). Capan-1 cells have been shown to be more sensitive to DNA damaging agents than other human cell lines were (11, 19). However, Capan-1 cells have many additional genetic alternations compared to these other human cell lines, whether the increased sensitivity of Capan-1 cells to genotoxic agents is caused by the lack of functional BRCA2 is not clear. The goals of this study are to investigate whether alternation of the expression of wild-type BRCA2 in human cell lines would alter the ability of these cells to repair their DNA damage. We have accomplished the Task 1 of this project, to establish Capan-1 derivatives that express wild-type BRCA2. We have completed the first part of Task 3 of this project, to investigate whether expression of wild-type BRCA2 alters the sensitivity of Capan-1 to DNA damaging agents. We are actively pursuing the Task 2, generation of MCF7 and MCF-12A derivatives that do not express BRCA2.

## Body

### 1. Characterization of wild-type BRCA2-expressing Capan-1 derivatives

We examine the sensitivity to DNA damaging chemicals of Capan-1 derivatives that expressed the wild-type BRCA2 and those did not. We chose methyl methanesulfonate (MMS), mitomycin C, etoposide and mitoxantrone for this study. The detail of this study is described in the legend of figure 1. We performed this study three times and with duplication experiment each time. Our results show that there is no apparent difference in the sensitivity to DNA damaging drugs between the Capan-1 cells expressing and not expressing wild-type BRCA2 (Figure 1). These results are consistent with the lack of reduced sensitivity to $\gamma$-radiation of BRCA2-expressing Capan-1 derivatives reported in last year's progress report. Thus, our study

demonstrates that expression of wild-type BRCA2 does not reduced the sensitivity of Capan-1 cells to DNA damaging agents.

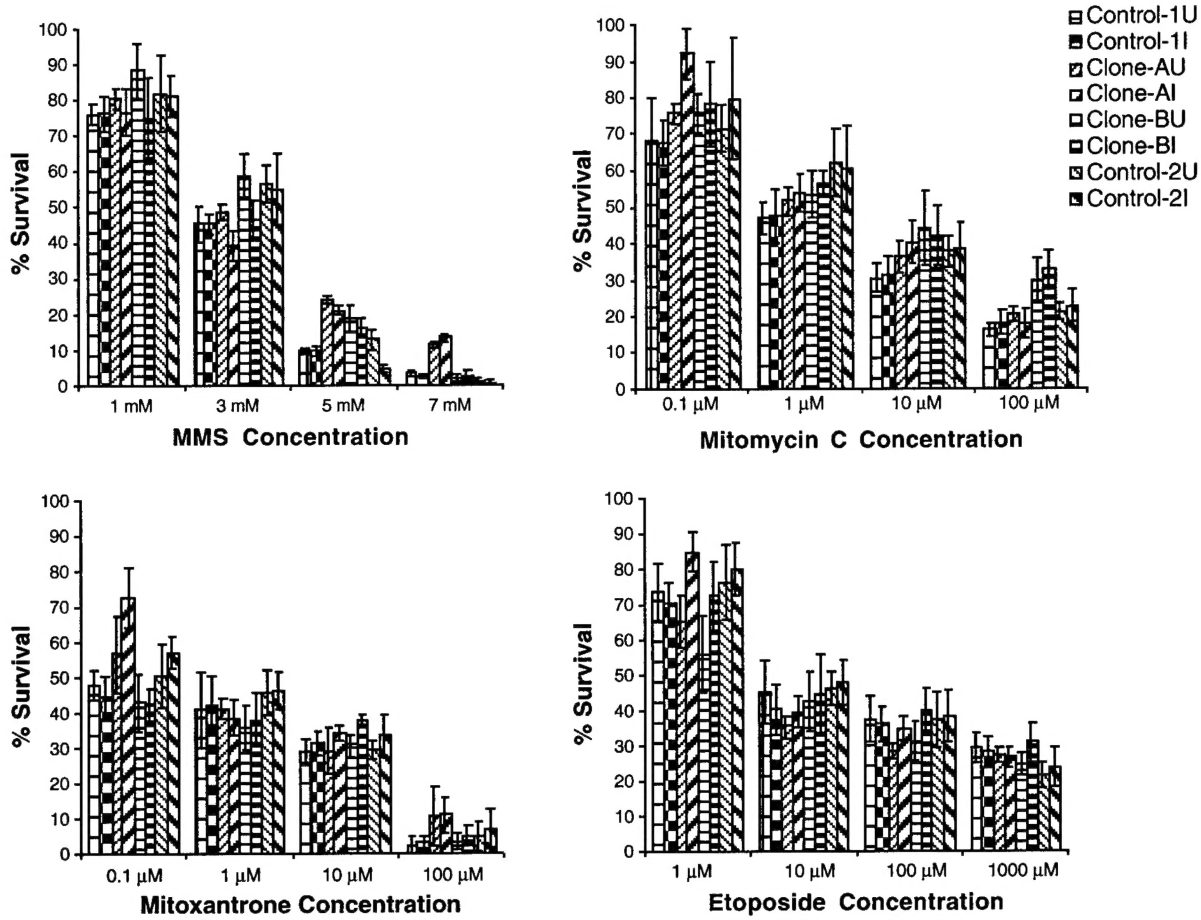


**Figure 1.** Sensitivity of Capan-1 derivatives to DNA damaging drugs. One thousand indicated cells were plated on each well of 96-well plate in the presence of tetracycline. Tetracycline was removed from (I) or maintained in (U) the media to regulate the expression of wild-type BRCA2 two days after cell plating. Another two days later, cells were treated with drugs as indicated. Cells were treated with MMS for one hour, washed then cultured in tetracycline-free (I) or tetracycline-containing (U) media for two days. For all other drugs, cells were treated for two days in tetracycline-free (I) or tetracycline-containing (U) media. Survived cells were measured by using MTT assay. The results of three duplicated experiments are shown.

2 Generate MCF7 and MCF-12A derivatives that do not express BRCA2

*A. Antisense approaches.* We have attempted to reduce BRCA2 level in cells by expressing a *BRCA2* cDNA fragment at antisense orientation. We used a 662 bp *BRCA2* cDNA fragment that contained 282 bp of 5' untranslated region and 380 bp of the most 5' coding region. MCF7 cells were transfected with plasmids expressing this *BRCA2* cDNA fragment at the antisense orientation under the control of either a constitutive cytomegaloviral early promoter or a tetracycline regulated promoter and stable transfectants were selected and isolated. We examined the BRCA2 protein level of more than 100 clones by immunoblotting but did not identify any clone that expressed reduced level of BRCA2.

*B. Somatic knockout.* We have isolated a genomic DNA clone that contained from intron 4 to intron 23 of *BRCA2*. We have generated a somatic knockout vector (Figure 2).

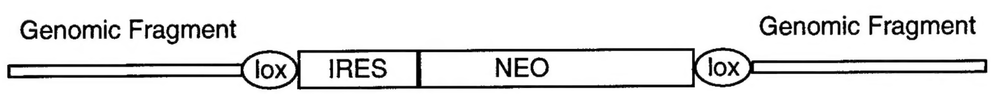


**Figure 2.** Vector for gene knockout.

*C. The small interfering RNA (siRNA) approach.* The siRNA approach has recently been demonstrated to be an effective method to reduce gene expression. For mammalian cells, this was demonstrated first by using synthetic RNA oligonucleotides (20, 21). More recently, several groups have shown that the expression of several genes could be reduced by transfecting cells with plasmids that express siRNA from RNA polymerase III promoters (22-30).

We have successfully reduced the BRCA2 level in MCF7 cells by transfecting them with two different synthetic siRNA targeting BRCA2 (Figure 3). In contrast, the BRCA2 level was not affected in cells transfected with siRNA targeting a different gene. We are currently

developing plasmids that expressing siRNA targeting BRCA2 and will use these plasmids to reduce BRCA2 in MCF7 and MSF-12A cells.


**Figure 3.** Reducing BRCA2 level by siRNA.

MCF7 cells were transfected with a synthetic siRNA targeting EBF3 (lane 1) or two different siRNAs targeting two different regions of *BRCA2* (lanes 2 and 3). Cell lysates isolated from transfected cells containing similar amount of total protein were resolved by SDS-PAGE on 6% (top panel) or 12% (bottom panel) gels and indicated proteins were detected by using immunoblotting (12, 31). For EBF3, the top two bands are two different forms of EBF3 translated from alternatively spliced mRNA whereas the bottom band is a nonspecific protein.

**Key Research Accomplishments**

- We have shown that expression of wild-type BRCA2 does not reduce the sensitivity of Capan-1 cells to DNA damaging agents.
- We have identified two different siRNAs that can reduce the expression of BRCA2.

**Reportable Outcomes**

- A paper showing the effect of expressing wild-type BRCA2 on the growth of Capan-1 cells has been published (Cancer Res., 62: 1311-1314, 2002).
- An abstract describing the results of characterization of wild-type BRCA2-expressing Capan–1 derivatives has been submitted to the third Era of Hope meeting.

## Conclusions

We have accomplished Task 1 of this project, generation of wild-type BRCA2-expressing Capan-1 derivatives. We have completed the first part of Task 3. We are also working on the Task 2, generation of MCF-12A and MCF7 derivatives that do not express wild-type BRCA2. We will continue working on Task 2 and Task 3 in the next year.

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**Appendix:**

Wang, S.-C., Shao, P., Pao, A. Y., Zhang, S., Hung, M.-C. and Su, L.-K. Inhibition of cancer cell growth by BRCA2. Cancer Res., 62: 1311-1314, 2002.

[CANCER RESEARCH 62, 1311–1314, March 1, 2002]

*Advances in Brief*

# Inhibition of Cancer Cell Growth by BRCA2[1]

**Shao-Chun Wang, Ruping Shao, Annie Y. Pao, Su Zhang, Mien-Chie Hung,[2] and Li-Kuo Su**

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## Abstract

**The breast cancer susceptibility gene *BRCA2* has been suggested to function as a "caretaker" of the genome. Cells without wild-type *BRCA2* are deficient in repairing DNA damage. However, whether *BRCA2* can also suppress oncogenesis by regulating cell proliferation remains to be determined. To address this question, the expression of wild-type BRCA2 protein was reconstituted, in an either constitutive or regulated manner, in the pancreatic cancer cell line Capan-1, which expresses only a mutant BRCA2. Expression of wild-type BRCA2 inhibited cell proliferation in culture and suppressed tumor growth in animals. Our results showed that, in addition to the DNA repair function, *BRCA2* also suppresses tumor development by inhibiting cancer cell growth.**

## Introduction

People carrying germ-line mutations of the breast cancer susceptibility gene *BRCA2* have increased risk for breast, ovarian, pancreatic, and other types of cancer (1–3). Tumors developed in heterozygous *BRCA2* mutation carriers are frequently associated with loss of heterozygosity at the *BRCA2* locus, a result consistent with a critical function of *BRCA2* in tumor suppression. BRCA2 has been suggested to be a "caretaker" and to play an important role in maintaining genomic integrity (4). Cells without a wild-type *BRCA2* gene are deficient in repairing the DNA damage caused by genotoxic agents, such as ionizing radiation (5–11). We and others have shown that the expression of BRCA2 is tightly regulated in a cell cycle-dependent manner, with an expression level low in $G_1$ phase and peaked in S-$G_2$ phases of cell cycle. Because the signaling of DNA damage repair is usually coupled with cell cycle progression, the question of whether BRCA2 can also regulate cell proliferation is intriguing and remains to be determined (12, 13). To address this question, we expressed the wild-type BRCA2 protein in Capan-1 cells. Capan-1 is a human pancreatic cancer cell line that expresses only a COOH-terminal truncated BRCA2 protein (14, 15). We established and characterized stable transfectants of Capan-1 cells that expressed wild-type BRCA2 either constitutively or through a tetracycline-regulated expression system. Our results showed that, in addition to the DNA repair function reported previously, BRCA2 also involved in the negative regulation of cell proliferation *in vitro* and tumor growth *in vivo*.

## Materials and Methods

**BRCA2 Expression Plasmids.** We isolated the cDNA for the entire coding region of *BRCA2* by RT-PCR.[3] Because of its large size, the *BRCA2* coding region was divided into four fragments for RT-PCR. Four to 10 clones of each amplified fragment were sequenced to identify those that did not contain any mutation resulting from the PCR reaction. These fragments were ligated sequentially together to obtain the full-length cDNA for *BRCA2*. The *Xho*I restriction site was engineered at both ends of the assembled *BRCA2* cDNA. To facilitate the assembly of the full-length coding cDNA of *BRCA2*, codon 798 was changed from CTC to CTT to create a *Hin*dIII restriction site; however, this change does not alter the encoded amino acid. To construct pCINBRCA2, the *BRCA2* cDNA was inserted at the *Xho*I site of an expression vector pCIN (16). To construct p236BRCA2, the pcDNA3 vector (Invitrogen, Calsbad, CA) was first modified by inserting a 236-bp fragment of the 5′ untranslated region of *BRCA2* between the *Kpn*I and *Not*I sites. The assembled full-length *BRCA2* cDNA was then inserted at the *Xho*I site of this plasmid. The 5′ untranslated region of *BRCA2* was obtained by RT-PCR using primers 5′-GGTACCGGTGGCGCGAGCTTCTGA-3′ and 5′-GCGGCCGCAACTACGATATTCCTCCAAT-3′.

**Generation of Wild-type BRCA2-expressing Capan-1 Derivatives.** The stable cell line CINBRCA2 was generated by transfecting Capan-1 cells with pCINBRCA2, and the cell clone 236BRCA2 was generated by transfecting Capan-1 cells with p236BRCA2. Plasmid DNA (10 $\mu$g) was mixed with the cationic liposome DC-Chol at a ratio of 1 $\mu$g DNA:13 nmol of DC-Chol (17). The DNA/liposome complex was then added to the cell culture dish and incubated for 16 h. Transfected cells were cultured for 3 days before subjected to G418 (500 $\mu$g/ml) selection. BRCA2TN, neoTN-1, and neoTN-2 were obtained by transfecting Capan-1 cells with tTA-IRES-Neo (18) together with a derivative of pUHD10–3 (19) that expressed *BRCA2* at 1:9 ratio using Lipofectamine Plus (Life Technologies, Inc.), then selected with G418. Resulted clones were screened for BRCA2 protein expression using anti-BRCA2 antibodies. Both neoTN-1 and neoTN-2 expressed the tetracycline-controlled transactivator but did not express wild-type BRCA2 protein (data not shown).

**Detection of the Expression of Exogenous BRCA2.** To detect the expression of the exogenous *BRCA2* RNA, a 524-bp *BRCA2* cDNA fragment containing codon 798 was amplified by RT-PCR and was digested with the restriction enzyme *Hin*dIII. The RT-PCR product of the exogenous *BRCA2* could be digested by *Hin*dIII to generate two fragments of 255 bp and 265 bp because of the presence of an engineered *Hin*dIII site. The RT-PCR product of the endogenous *BRCA2* RNA lacked this *Hin*dIII site and remained intact as a 524-bp fragment.

Immunoblotting was used to detect BRCA2 protein. Cells were harvested and lysed in ice-cold NETN [150 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl (pH 8.0), and 0.5% NP40] or radioimmunoprecipitation assay buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% NP40] buffer. Total cell protein extracts were quantified by the Bradford assay (Bio-Rad, Hercules, CA.). Equal amounts of lysate protein (60–120 $\mu$g) were separated by SDS-PAGE and blotted to polyvinylidene fluoride membrane (Bio-Rad). Proteins were then identified using BRCA2 antibodies N61 (15) or Ab-2 (Oncogene Science, Cambridge, MA).

**Determination of Cell Growth Rate.** Thymidine incorporation assay was used to measure DNA synthesis. Cells (250) were plated in each well of a 96-well plate and cultured for 48 h, then incubated with 1 $\mu$Ci of [methyl-[$^3$H]] thymidine (Amersham, Arlington Heights, IL.) in the presence of serum for 16 h. Cells were harvested, and the amount of incorporated [methyl-[$^3$H]] thymidine was measured with a scintillation counter (Beckman). To measure the rate of cell number increasing, 2000 cells were plated in each well of a 96-well plate. Cell number of the culture at different days after plating was determined by using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay. Clonogenic assay was also used to measure the growth rate of cells. Cells were plated in a six-well plate at a density of 1000 cells/well and allowed to grow for 2.5 weeks with media changed every 2 days. For tetracycline-regulated Capan-1 derivatives, cells were cultured in the tetracy-

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[3] The abbreviation used is: RT-PCR, reverse transcriptase-PCR.

cline-containing media all of the time or in the tetracycline-free media beginning 2 days after plating.

**Tumorigenicity Assay and Culture of Tumor Cells.** Cells were harvested by trypsinization, washed with PBS, then suspended in PBS at the density of $1 \times 10^7$ cells/ml. Cell suspension was injected s.c. into both flanks of female nude mice of 6–8 weeks of age. Tumor volumes were determined by external measurement in two dimensions and calculated using the equation $V = (L \times W^2) \times 0.5$, where $V$ is volume, $L$ is length, and $W$ is width. To recover the cells from the 236BRCA2-derived tumor, the tumor was resected, chopped, and digested with trypsin, then plated for culturing. Individual colonies and a pool of the tumor cells were obtained. Only cells of the early passages (two to six passages) were used for molecular characterization. Animal care was performed in accord with institution guidelines.

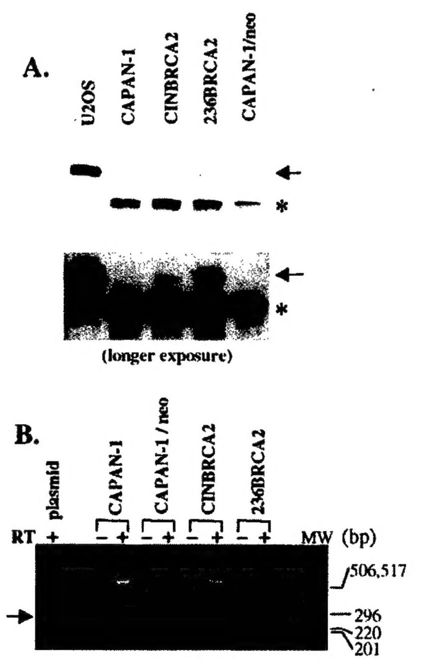
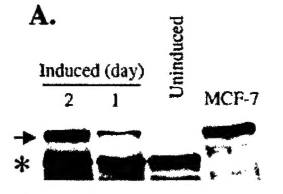



Fig. 1. Generation of constitutive wild-type BRCA2-expressing Capan-1 derivatives. *A*, expression of wild-type BRCA2 protein. Proteins isolated from a positive control cell line (U-2 OS) that expressed endogenous wild-type BRCA2, two negative control cell lines (*Capan-1* and *Capan-1/neo*), and the two *BRCA2*-expressing clones (*CINBRCA2* and *236BRCA2*) were separated using SDS-PAGE on a 6% gel. BRCA2 proteins were detected by the monoclonal antibody N61 that recognized the $NH_2$-terminal region of the BRCA2 (15). *Arrows*, the full-length BRCA2; *, the endogenous truncated BRCA2 in Capan-1 cells. *B*, expression of exogenous *BRCA2* mRNA. *BRCA2* RNA isolated from the parental cells (*Capan-1*), the vector-transfected cells (*Capan-1/neo*), and the two *BRCA2*-expressing derivatives (*236BRCA2* and *CINBRCA2*) was amplified using RT-PCR. The RT-PCR products were digested with *Hin*dIII and resolved on an agarose gel. The RT-PCR product of the exogenous *BRCA2* could be digested by *Hin*dIII to generate two fragments of 255 and 265 bp because of the presence of an engineered *Hin*dIII site. The RT-PCR product of the endogenous *BRCA2* RNA lacked this *Hin*dIII site and remained intact as a 524-bp fragment. The *Hin*dIII-digested PCR product from a plasmid carrying the *BRCA2* cDNA was included as a positive control. *Arrow*, the digested small fragments, which were not resolved in this gel. *RT*, reverse transcriptase.




C.

| | subG0 | G0/G1 | S | G2/M |
|---|---|---|---|---|
| neoTN-1 (U) | 1.94 | 54.1 | 28.1 | 17.8 |
| neoTN-1 (I) | 1.60 | 58.2 | 25.7 | 16.1 |
| neoTN-2 (U) | 2.11 | 47.3 | 32.0 | 20.6 |
| neoTN-2 (I) | 1.82 | 53.5 | 26.7 | 19.8 |
| BRCA2TN (U) | 2.81 | 62.2 | 25.9 | 11.9 |
| BRCA2TN (I) | 1.63 | 56.1 | 28.0 | 15.9 |

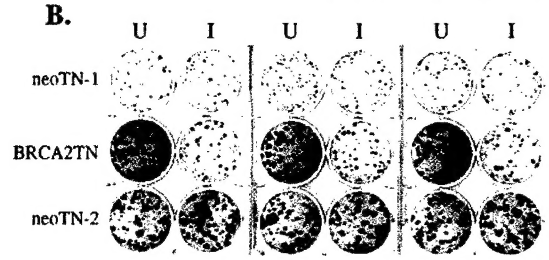
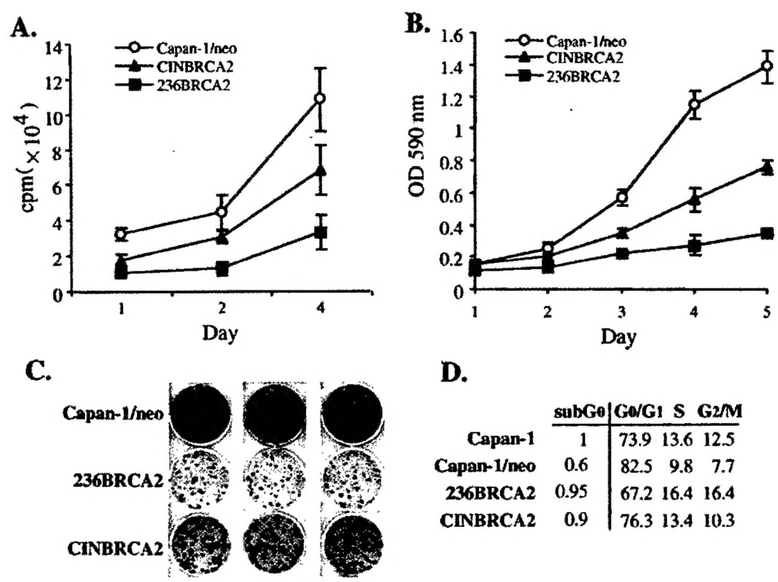



Fig. 3. Inhibition of Capan-1 growth by inducible wild-type BRCA2 expression. *A*, tetracycline-regulated BRCA2 expression in BRCA2TN. Lysates were prepared from BRCA2TN cells grown in the presence (*Uninduced*) or absence (*Induced*) of tetracycline for 1 or 2 days as indicated. The lysate of MCF-7, a breast cancer cell line that expresses wild-type BRCA2 protein (15), was included as a positive control. BRCA2 was detected by immunoblotting using N61 antibody. *Arrow* and *, the full-length BRCA2 and endogenous truncated BRCA2 in Capan-1 cells, respectively. *B*, suppression of BRCA2TN growth by the wild-type BRCA2. Cells (1000) from each of the indicated Capan-1 derivatives were plated in each well of six-well plates in medium containing tetracycline. Cells were grown for 2.5 weeks, either in media containing tetracycline (*U*) or in tetracycline-free media (*I*) beginning 2 days after plating. The resulting colonies were stained with *crystal violet*. The results shown are from a triplicate experiment. *C*, flow cytometry analysis of the BRCA2 inducible, as well as the control cell lines. *Numbers*, the percentage of cells in each cell cycle stage. Data shown are derived from a representative experiment. Data shown are derived from a representative experiment. The percentage of each cell cycle stage added up to be 100% and was determined independently from the $subG_0$ measurement. *U*, uninduced; *I*, induced.




D.

| | subG0 | G0/G1 | S | G2/M |
|---|---|---|---|---|
| Capan-1 | 1 | 73.9 | 13.6 | 12.5 |
| Capan-1/neo | 0.6 | 82.5 | 9.8 | 7.7 |
| 236BRCA2 | 0.95 | 67.2 | 16.4 | 16.4 |
| CINBRCA2 | 0.9 | 76.3 | 13.4 | 10.3 |

Fig. 2. Inhibition of Capan-1 cell growth *in vitro* by constitutive wild-type BRCA2 expression. *A*, cell growth assay using [$^3$H]thymidine incorporation. The results shown are from a quadruplet assay. *B*, determination of cell growth by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide assay. The results shown are from a quadruplet assay. *C*, clonogenic assay for cell growth. Cells (1000) from the indicated Capan-1 derivatives were plated in each well of six-well plates. The cells were allowed to grow for 2.5 weeks, and the resulting colonies were stained with *crystal violet*. The results shown are from a triplicate experiment. *D*, flow cytometry analysis of BRCA2 transfectants. *Numbers*, the percentage of cells in each cell cycle stage. Data shown are derived from a representative experiment. The percentage of each cell cycle stage added up to be 100% and was determined independently from the $subG_0$ measurement.

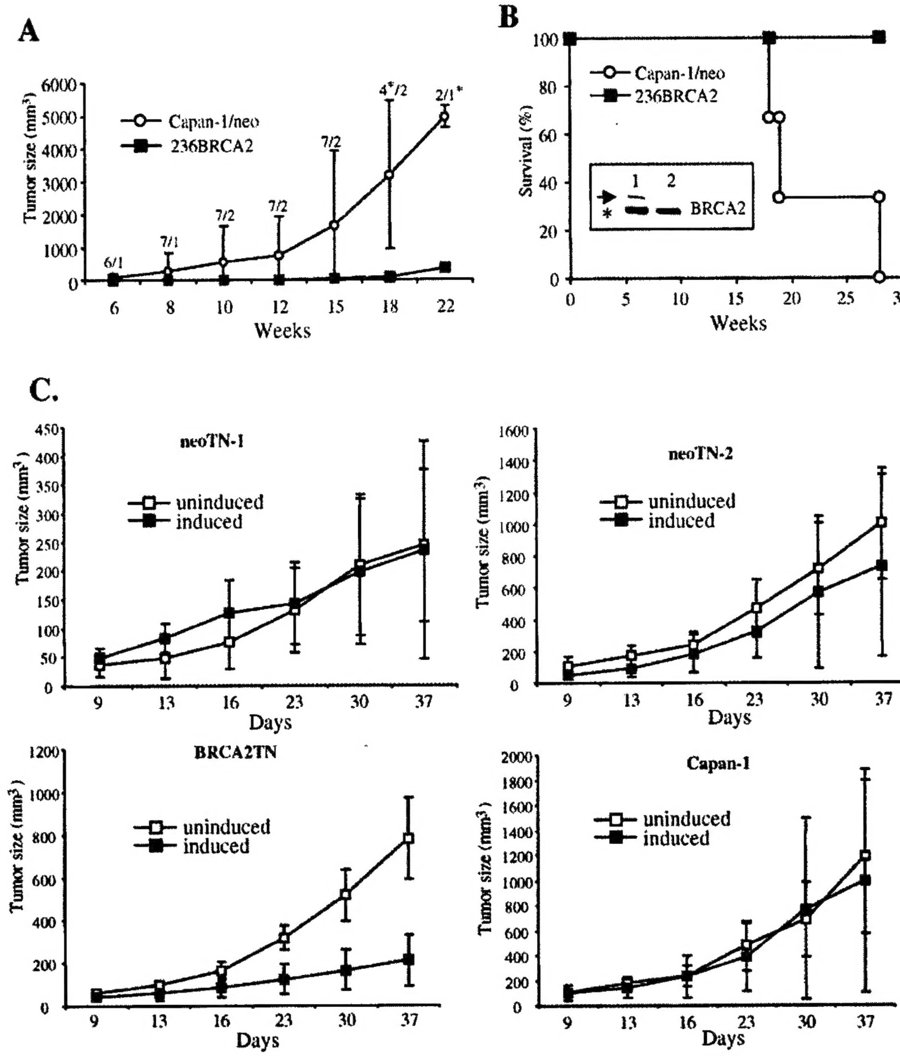



Fig. 4. Inhibition of Capan-1 cell growth *in vivo* by the wild-type *BRCA2* gene. *A*, capan-1/neo or 236BRCA2 cells (1 × $10^5$) were inoculated s.c. into both flanks of each mouse (five mice in each group), and the tumor number and volume were determined starting 6 weeks after inoculation. The number of tumors resulting from each cell line is shown at each time point (*Capan-1/neo /236BRCA2*). One tumor in each group was lost for unknown reasons, and both tumors were very small and grew slowly. *, the time of the loss. Additionally, the number of Capan-1/neo tumors was reduced later in the experiment because of the death of animal. *B*, survival curves of the mice bearing Capan-1/neo- or 236BRCA2-derived tumors as described in *A*. The only remaining 236BRCA2-derived tumor was dissected at week 28, and tumor cells were recovered. *Inset*, the expression of wild-type BRCA2 protein was decreased in the tumor-derived cells (*Lane 2*) compared with that in the original 236BRCA2 cells (*Lane 1*). *Arrow* and *, the full-length BRCA2 and endogenous truncated BRCA2 proteins in Capan-1 cells, respectively. The truncated BRCA2 protein confirmed the Capan-1 origin of the recovered cells. *C*, induction of BRCA2 expression inhibited the growth of the Capan-1-derived tumors. Ten nude mice were s.c. inoculated with 1 × $10^6$ of the indicated cells on each side of their flanks. Five mice in each group had been fed with doxycycline (0.2 mg/ml) in drinking water for 5 days before the inoculation. The mice were fed continuously with water containing doxycycline (□) or without doxycycline (■). The tumor volume was measured on the indicated days.

## Results and Discussion

To investigate the possible role of wild-type BRCA2 in regulating cell growth, we generated Capan-1 derivatives that express wild-type BRCA2. We did not use approaches that transiently express BRCA2 in Capan-1 cells, such as transient transfection or virus-mediated gene transfer, because these approaches would not allow us to study the phenotypes of BRCA2-expressing Capan-1 cells for a long period of time. In addition, the Capan-1 cell line is known to have very low transfection efficiency (7), and the size of *BRCA2* cDNA (~10 kb) is beyond the packaging capacity of virus when using commonly used recombinant viral vectors.

We first obtained two Capan-1 derivatives (236BRCA2 and CINBRCA2) that constitutively expressed wild-type BRCA2 after screening ~400 G418-resistant clones by immunoblotting (Fig. 1*A*). The level of full-length BRCA2 protein in clone 236BRCA2 was higher than that of CINBRCA2. The expression of wild-type BRCA2 protein was confirmed by using immunoprecipitation followed by immunoblotting with different BRCA2 antibodies (data not shown). The expression of the exogenous *BRCA2* RNA in these two clones was also demonstrated by RT-PCR followed by *Hin*dIII digestion (Fig. 1*B*). The growth rate of these two BRCA2-expressing clones correlated with the expression levels of wild-type BRCA2 protein, and both clones grew slower than the parental Capan-1 cells and a control Capan-1 derivative (Capan-1/neo), which was transfected with an empty vector (Fig. 2). These results suggested that high-level expression of BRCA2 in the Capan-1 cells was not compatible with cell growth and might partially explain the difficulty in obtaining Capan-1 derivatives that constitutively expressed wild-type BRCA2 protein.

To rule out the possibility that the reduced growth rate of BRCA2-expressing derivatives was simply because of clonal variation and confirm that the cell growth suppression was the consequence of expressing wild-type BRCA2, we established a Capan-1 derivative (BRCA2TN) that expressed wild-type BRCA2 protein under the regulation of tetracycline (Fig. 3*A*). The growth of BRCA2TN was suppressed significantly when the wild-type BRCA2 protein was induced to express in the absence of tetracycline (Fig. 3*B*). The growth inhibition was unlikely simply because of the activation of the tetracycline-controlled transactivator, because the growth of two control cell lines (neoTN-1 and neoTN-2) that did not express BRCA2 but expressed functional tetracycline-controlled transactivator (data not shown) was not affected by the removal of tetracycline (Fig. 3*B*).

Capan-1 cells are highly tumorigenic in nude mice. In a preliminary experiment, we found that expression of wild-type BRCA2 suppressed tumorigenesis, and the level of suppression correlated with the level of BRCA2 (data not shown). This observation was confirmed in a subsequent experiment. Of 10 inoculations, 236BRCA2 cells re-

sulted in only two slow-growing tumors, whereas Capan-1/neo cells resulted in six vigorously growing tumors (Fig. 4*A*). All of the mice inoculated with 236BRCA2 cells were still alive 28 weeks after the inoculation, whereas all of the mice bearing Capan-1/neo-derived tumors died in ≤28 weeks of the inoculation (Fig. 4*B*). The expression of wild-type BRCA2 protein was undetectable in cells recovered from the 236BRCA2-derived tumor. This result suggested that loss of wild-type BRCA2 protein expression might be necessary for the growth of such tumors (Fig. 4*B*, *inset*).

The inhibition of tumor growth by BRCA2 was confirmed when BRCA2TN cells were studied. The growth rate of these cells was reduced significantly in mice that the wild-type BRCA2 protein was induced to express than in mice that the expression was repressed. The inhibition of tumor growth was not because of the activation of tetracycline-regulated transactivator or lack of doxycycline because there was no difference in the growth of control cell lines (Capan-1, neoTN-1, and neoTN2) between the two groups of mice (Fig. 4*C*).

By reconstituting wild-type *BRCA2* expression in the *BRCA2* mutant cell line Capan-1, we demonstrated that the expression of wild-type BRCA2 protein suppressed the growth of Capan-1 cells *in vitro* and *in vivo*. Our results strongly suggest that, in addition to guarding the genomic integrity as reported previously (5–11), regulation of cell proliferation contributes to the tumor suppression function of *BRCA2*. Because the *p53* gene is mutated in Capan-1 cells (20), this cell-growth inhibition likely occurs through a p53-independent mechanism. The Rb pathway is also not required for growth suppression by BRCA2 because the Rb cell cycle regulation pathway is not functional in Capan-1 cells (21). Flow cytometry analysis of the constitutive BRCA2 transfectants, as well as the inducible BRCA2 clone, did not show an increased sub-$G_0$-$G_1$ cell population, nor significant abnormality of cell cycle distribution (Fig. 2*D* and 3*C*). Therefore, the decreased growth rate of these two clones was unlikely to have resulted from increased cell death or arrest of cell cycle progression at certain stages. Additional investigations will be necessary to understand the mechanism of regulating cell proliferation by BRCA2.

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